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Kinetic cutinase-catalyzed esterification of caproic acid in organic solvent system

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ABSTRACT

Practical application of any chemical reaction requires the knowledge of its kinetics; in particular if one wishes to be able to describe a chemical reactor over an extended range of reaction conditions or if one intends to optimize the reaction conditions, a suitable kinetic model must be obtained. In order to ensure that the model is applicable over a wide range of experimental conditions it should be based on a mechanistic scheme describing the fundamental steps involved in the reaction; the development of these kind of models can also be used to provide insight into the processes that are taking place.

A kinetic study, using experiments carried out in a batch stirred reactor, has been made for the enzymatic esterification of caproic acid with ethyl alcohol catalyzed by *Fusarium solani pisi* cutinase. Different acid and alcohol concentrations (whilst also varying the acid/alcohol molar ratio) were tested and the results were used to identify the best reaction scheme to describe the results obtained over an extended range of conditions. Several different approaches were used to identify the most adequate mechanistic model, namely by resorting to the quasi stationary state and the rate-limiting hypothesis. The main kinetic characteristics observed in esterification reaction were found to follow an ordered Ping-Pong Bi–Bi mechanism but different modifications were used o ensure that the kinetic model was applicable over the entire range of experimental conditions that were covered.

1. Introduction

Studies of various enzymes (mostly lipases and esterases) in enzymatic synthesis of short chain acid esters, compounds, which have very significant application in cosmetic, pharmaceutical and food industries, increased last few decades [1–4]. Enzyme catalyzed esterification is an interesting option when compared to chemical synthesis as it has the advantages of being able to be carried-out under mild reaction conditions, and to ensure the high quality and purity of the products; also enzymes have been considered as natural components by food regulatory agencies [5–7].

Fusarium solani pisi cutinase activity in hydrolysis, esterification and transesterification has been extensively exploited in recent years and several applications in different industrial fields have been proposed [8]. Numerous report of using cutinases in different reaction media, often dissolved in aqueous solution but also suspended as a powder or immobilized, have been reported [8,9]. Immobilization has been commonly obtained by adsorption onto solid supports [10–19] or encapsulation in reverse micelles of sodium bis(2-ethylhexyl)sulfosuccinate (AOT) [20–23], phosphatidylcholine [24], or cetyltrimethylammonium bromide (CTAB) [25]. The cutinase was used in fundamental studies on the hydrolysis of triglycerides [26], transesterification [15–17], in studies of esterification reactions [27–31] and in order to clarify cutinase mechanism of reaction regarding stereo-selectivity and specificity [32,33]. The understanding of the reaction kinetics is of importance not only to explain in the analysis of the mechanism of the reaction, but also because the information about the rate of product formation and changes in experimental system are essential for the design of appropriate reactor and later industrial scale up.

Modeling of cutinase-catalyzed reaction for transesterification [18,21,22] and esterificaton reaction [24] has been reported. A Ping-Pong Bi–Bi mechanism with competitive inhibition by the alcohol was proposed by Serralha et al. [18] for the alcoholysis of butyl acetate with hexanol in isooctane catalyzed by cutinase immobilized on NaY zeolite. Ping-Pong Bi–Bi mechanism was also proposed for the same reaction in AOT/isooctane reverse micellar system by Carvalho et al. [22] and by Sebastião et al. [21] and Pinto-Sousa et al. [24] for the esterification reaction in phosphatidylcholine reversed miccelar system. Although these representative examples of research work focused on the kinetic analysis of cutinase-catalyzed reaction, mostly performed on transesterification and esterification reactions in reversed micellar systems, no detailed kinetic model for esterification reaction in organic solvent, namely in *iso*-octane, by lyophilized cutinase, was presented, despite the catalytic potential of cutinase in organic solvent system. However,

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Nomenclature

Ac	acid
Al	alcohol
Es	ester
E	free enzyme
$[E]_t$	total enzyme concentration (mM)
EAc	enzyme–acyl complex
EAc2	enzyme–acyl complex with two bound acid molecules
EAc3	enzyme–acyl complex with three bound acid molecules
EAl	enzyme–alcohol complex
K_{a1}, K_{a2}, K_{a3}, K_{b1}, K_{b2}, K_{b3}	equilibrium constants
K_i	inhibition coordination constant
k_{a1}, k_{a2}, k_{a3}, k_{b1}, k_{b2}, k_{b3}	rate constants ($mM^{-1}\ min^{-1}$)

the development of a detailed kinetic model that is capable of describing the reaction over an extended range of reactions conditions is of paramount importance for practical applications, namely for the optimisation of reaction systems.

Cutinases are a group of enzymes that can be considered as a link between esterases and lipases. As small carboxylic ester hydrolases, the sub-family of cutinases consists of 20 members based on amino-acid sequence similarity, which display hydrolytic activity on cutin polymers and efficiently hydrolyze soluble esters and emulsified triacylglycerols. Cutinase belongs to the family of serine hydrolases containing its catalytic serine centre at the middle of a sharp turn between a β-strand and a α-helix [14,34]. The *F. s. pisi* catalytic triad, *Ser-120*, *Asp-175* and *His-188*, is accessible to the solvent and it can possibility to accommodate different substrates. The *Asp-175* hydrogen binds to *His-188*, thus promoting the interaction of the imidazole ring with *Ser-120*. The histidine acts as a base, deprotonating the serine to generate a very nucleophilic alkoxide (–O–) group (Fig. 1). The serine in the active centre of the enzyme is a very strong nucleophile, which attacks the carbonyl group of the acid, forming a stable tetrahedral intermediate acyl–enzyme complex. The acyl–enzyme complex is stabilized by the oxyanion hole [12]. Water is then released and the structure reverts to the planar carbonyl flat plane acyl–enzyme intermediate. The alcohol acts afterwards as a new nucleophile and links to the tetrahedral intermediate. Subsequently, as the final step, the resolution of tetrahedral complex yields the ester and the free enzyme. This reversible reaction was shown to follow a Ping-Pong Bi–Bi mechanism [8,35].

In previous work made by de Barros et al. [30] *F. s. pisi* cutinase expressed in *Saccharomyces cerevisiae* showed a high potential for the synthesis of short chain ethyl esters in *iso*-octane, an organic solvent recognized as a safe ingredient for use in the food and beverage industrial processes by FDA. Among the advantages of using the organic solvent media for enzymatic ester synthesis are the increased solubility of non-polar substrates and products and the shifting the thermodynamic equilibrium of the reaction to favour ester synthesis over hydrolysis. Log *P* (the partition coefficient between water and 1-octanol) is generally used to describe the solvent hydrophobicity. It has been reported that solvent with intermediate log *P* (around 4) are suitable for esters synthesis [36]. Log *P* of *iso*-octane is 4.5 and by previous work it was shown that it favours the esterification reaction, reason for which it was chosen for this work.

Since mechanism based kinetic models are usually better for interpolating and extrapolating purposes, the goal of this study is to estimate the kinetic parameters of this system in terms of a Ping-Pong Bi–Bi model and to analyse possible refinements that can be introduced in this model to improve the description of the reaction system with the aim of obtaining a model that can be used over a sufficiently wide range of operating conditions so as to allow the optimization of a bioreactor for this reaction.

As a model system the production of ethyl caproate from caproic acid and ethanol has been used. Ethyl caproate is a flavour compound that is incorporated in a wide range of aromas such as: apple, apple green apple, banana, beer, butter, cognac, herbal, pineapple and wine [37].

2. Materials and methods

2.1. Enzyme and chemicals

F. s. pisi cutinase wild-type was biosynthesized by recombinant *S. cerevisiae* SU50 strain as described by Calado et al. [38].

Caproic acid (C_6) (99.0%, Fluka, Germany) and ethanol abs. (VWR, Germany) were used for ester synthesis, while *iso*-octane (99.5%, Fluka, Germany) was used as organic solvent and n-decane (VWR, Germany) was used as an internal standard for gas chromatography (GC). Sodium sulfate anhydride (Acros, Geel, Belgium) was used to dry *iso*-octane as organic media before and after esterification reactions. Saturated salt solution of sodium chloride (Panreac, Spain) was used for equilibration of enzyme and substrates. All other chemicals used were of analytical grade.

2.2. Purification and lyophilization of cutinase

The isolation and purification of cutinase excreted by recombinant *S. cerevisiae* SU50 strain were carried out by expanded bed adsorption (EBA) [39].

The pool of elution fractions exhibiting the highest cutinase activity was firstly dialyzed against distilled water and then frozen at −80 °C and lyophilized (B.BRAUN Biotech. International CHRIST Alpha 2-4) overnight. Lyophilized cutinase preparations were characterized by measuring the cutinase esterolytic activity and protein content [40–42] and stored at −20 °C before used in esterification reactions.

2.3. Characterization of the cutinase preparations

The cutinase estereolytic activity was assayed using a spectrophotometric method based on monitoring the hydrolysis of p-nitrophenylbutyrate (p-NPB) to nitrophenol (p-NP), a yellow compound easily identify and quantify by the absorbance at 400 nm [40]. One unit of cutinase estereolytic activity was defined as the amount of enzyme required to convert 1 μmol of p-NPB to p-NP per 1 min, at 30 °C and pH 8. The extinction coefficient of p-NP was considered to be 1.84×10^4 ($M^{-1}\ cm^{-1}$), as indicated by the supplier (Sigma).

The protein concentration was determined by the method of PEARCE (BCA assay) with reference to a standard, the Bovine Serum Albumin (BSA) (Merck) [42].

Specific activities of lyophilized cutinase preparations were of 170 U mg^{-1}. These enzyme samples when loaded in SDS electrophoresis gel showed a single band of 22 kDa [41].

2.4. Methods for monitoring substrate and ester concentrations

The concentrations of ethanol, caproic acid and ethyl caproate were determined using a Hewlett-Packard model 5890 gas chromatograph, equipped with a flame ionization detector (FID). A WCOT Fused Silica coating CP Chirasil-Dex CB column, 25 m × 0.25 mm, DF = 0.25 (Varian Inc.) was used to separate the components in the reaction mixture. n-Decane was used as an internal standard in the computation of ethyl caproate and respective